



PATENT

ATTORNEY DOCKET NO: 01997/201006

IDENTIFICATION AND CHARACTERIZATION OF A GENE WHICH PROTECTS
CELLS FROM PROGRAMMED CELL DEATH AND USES THEREFOR

5 Cross Reference To Related Applications

This application is a continuation-in-part of U.S.S.N. 09/234,186, filed on January 20, 1999, which in turn is a divisional of U.S.S.N. 08/801,248, filed on February 19, 1997, now abandoned, which in turn is a continuation of U.S.S.N. 08/288,295, filed on August 10, 1994, now abandoned, which in turn is a
 10 divisional of U.S.S.N. 07/927,681, filed on August 10, 1992, now abandoned, and, which in turn is a continuation-in-part of U.S.S.N. 07/898,933, filed on June 12, 1992, now abandoned, the disclosures of which are hereby incorporated by reference.

Government Funding

15 Work described herein was supported by grant number GM24663 from the U.S. Public Health Service. The United States government has certain rights in the invention.

Background of the Invention

Cell death is a fundamental aspect of animal development. A
 20 considerable proportion of the cells that are generated die during the normal development of both vertebrates (Glucksmann, Biol. Rev. Cambridge Philos. Soc. 26:59-86 (1951)) and invertebrates (Truman and Schwartz, Ann. Rev. Neurosci. 7:171-188 (1984)). Cell death plays a role in morphogenesis (e.g., of the eye, secondary palate, heart, nervous system and limbs in vertebrate embryos),
 25 metamorphosis (e.g., in moths and other insects), and tissue homeostasis (e.g., of epithelial linings and the thymus), as well as in neuron selection during the establishment of synaptic connections and in sexual dimorphism (reviewed by Ellis et al., Ann. Rev. Cell Biol. 7:663-698 (1991)). Cell

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death which occurs as a part of normal development will be referred to herein as physiological cell death.

Besides physiological cell death, cell death may occur as a pathological manifestation of disease, in which case it will be referred to herein as pathological cell death (see review by Trump and Mergner (1974), in: The Inflammatory Process, vol. 1, 2nd ed. (eds. Zweifach et al.), Academic Press, New York, pp. 115-257). Cell death can result from a variety of injuries to the cell, including toxins, ischemia (lack of blood supply), hypoxia (lack of oxygen) and infectious agents, as well as from genetic mutations. The major clinical aspects of most degenerative diseases are a consequence of cell death. For example, Huntingtons's disease, Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis are marked by degeneration of neurons, while Duchenne muscular dystrophy is characterized by muscle degeneration. In addition, some cancers are thought to be caused by a defect in cell death processes. Thus, understanding and preventing cell death can be viewed as one of the major goals of biomedical research.

The simple and invariant anatomy and development of the nematode Caenorhabditis elegans have made it an attractive system for the study of cell death. Because C. elegans is small, cellularly simple and transparent, Nomarski differential interference microscopy can be used to observe individual cells throughout development. As a result, the complete cell lineage of C. elegans, from zygote to adult, has been elucidated (Sulston and Horvitz, Dev. Biol. 82:110-156 (1977); Kimble and Hirsh, Dev. Biol. 70:396-417 (1979); Sulston et al., Dev. Biol. 100:64-119 (1983)).

Cell death is an important component of the development of C. elegans: during the development of the

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adult hermaphrodite, the generation of 816 nongonadal cells is accompanied by the generation and subsequent deaths of an additional 131 cells. Cell death appears to be an integral part of the differentiation of a variety of tissues. The pattern of cell deaths is essentially invariant among different animals, i.e., the same set of cells die at the same developmental time. In addition, a vast majority of cell deaths in C. elegans does not appear to be initiated by interaction with surrounding cells or diffusible factors.

Genetic analysis has identified many genes that affect programmed cell death in C. elegans (reviewed by Ellis et al. (1991) supra). The activities of two genes, ced-3 and ced-4, seem to be required for the onset of almost all C. elegans programmed cell deaths (Ellis and Horvitz, Cell 44:817-829 (1986)). Mutations in ced-3 and ced-4 block essentially all programmed cell deaths. In ced-3 and ced-4 mutants, cells that normally undergo programmed cell death instead survive, differentiate and even function (Ellis and Horvitz (1986) supra; Avery and Horvitz, Cell 51:1071-1078 (1987); White et al., Phil. Trans. R. Soc. Lond. B. 331:263-172 (1991)). Genetic analyses indicate that ced-3 and ced-4 genes most likely act within dying cells; this suggests that of these genes are expressed within dying cells and either encode cytotoxic molecules or control the activities of cytotoxic molecules (Yuan and Horvitz, Dev. Biol. 138:33-41 (1990)).

Relatively little is known about the mechanism of cell death. Initiation of cell death occurs in response to a variety of signals. External injuries and cytotoxic agents cause cells to die. Endocrine signals trigger cell death during insect metamorphosis, thymocyte death and regression of the prostate in the

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male rat after castration. Lack of neuronal growth factors is suspected to be the cause of certain neuronal cell deaths during vertebrate development and may also be the cause of cell deaths in certain neurodegenerative diseases. A specific protein, Mullerian inhibiting substance, is responsible for the regression of the Mullerian duct during the development of male mammals. In addition, genetically programmed cell deaths which occur apparently autonomously of cell-cell interaction or diffusible factors are observed in C. elegans and other invertebrates. (Truman and Schwartz, Neuro. Comm. 1:66-72 (1982); Cohen and Duke, J. Immunol. 132:38-42 (1984); Isaacs, Prostate 5:545-557 (1984); Martin et al., J. Cell. Biol. 106:829-844 (1988); Oppenheim and Prevette, Neurosci. Abstr. 14:368 (1988); Beal et al., Nature 321:168-171 (1986); Birkmayor and Hornykiewicz, Advances in Parkinsonism, Fifth International Symposium on Parkinson's Disease, Vienna, Roche, Basle, 1976; Lagsto et al., Science 219:979-980 (1983); Rossor, Lancet 2:1200-1204 (1982); Biel et al., Science 229:289-291 (1985); Cosi et al., in: Advances in Experimental Medicine and Biology, vol. 209, Plenum Press, New York, 1987; Bonilla et al., Cell 54:447-452 (1988); Picard and Josso, Biomedicine 25:147-150 (1976)).

Cell deaths also vary morphologically. Two major categories of cell deaths have been established based on morphological features (Kerr et al., Br. J. Cancer 26:239-257 (1972)). The first type of cell death, called necrosis, is characterized by cellular swelling, rupture of plasma and internal membranes, and eventual leakage of cellular contents into the extracellular space. The second, called apoptosis, involves progressive condensation of cytoplasm and nuclear

chromatin and eventual fragmentation of cellular membranes into 'apoptotic bodies', which are usually digested by macrophages or adjacent epithelial cells. Necrosis is most often a manifestation of certain pathological conditions, e.g., injury by complement (Hawkins et al., Am. J. Pathol. 68:255-288 (1972)), hypoxia (Jennings et al., Am. J. Pathol. 81:179-198 (1975)), or exposure to a variety of toxins (McLean et al., Int. Rev. Exp. Pathol. 4:127-157 (1965)). In contrast, apoptosis is usually associated with physiological conditions, e.g., embryogenesis (Bellari, J. Anat. 95:54-60 (1961); Saunders, Science 154:604-612 (1966)) and metamorphosis (Truman, Ann. Rev. Neurosci. 7:171-188 (1984)). Interestingly, morphological features of physiological cell death in C. elegans resemble, in general, those of apoptosis in vertebrates (Ellis et al., Ann. Rev. Cell Biol. 7:663-698 (1991)). However, deviations from the standard descriptions of necrosis and apoptosis are often observed. It is uncertain whether this morphological classification reflects real differences in underlying mechanisms of cell death.

Although the initiation and morphology of cell death vary, there is evidence which suggests that most physiological and some pathological cell deaths may share a common feature involving the activation of cell death genes. The existence of a genetic cell death program in a variety of organisms is suggested by the observation that protein and RNA synthesis inhibitors can prevent or delay a variety of cell deaths (insect metamorphosis, prostate regression, vertebrate neuronal cell death and thymocyte cell death) (Lockshin, J. Insect Physiol. 15:1505-1516 (1969); Stanasic et al., Invest. Urol. 16:19-22 (1978); Martin et al. (1988) supra; Oppenheim and Prevette (1988) supra; Cohen and

Duke (1984) supra). New RNA and protein species have been found after the initiation of cell death in the rat prostate after castration (Buttayan et al., Molecular Endocrinology 2:650-657 (1988); Lee et al., Prostate 7:171-185 (1985)). Thus, a better understanding of the mechanisms of cell death would have wide biological application and provide a basis for altering or controlling the process.

Summary of the Invention

10 The present invention relates to genes, referred to herein as cell death-protective genes, which function to protect cells against programmed cell death by antagonizing the activity of genes which cause cell death. As described herein, Applicants have identified
15 what appears to be a key or master regulatory gene whose activity determines whether a cell survives or undergoes cell death.

Specifically, a cell death-protective gene from the nematode Caenorhabditis elegans, called ced-9, has
20 been identified, sequenced, and characterized. ced-9 is essential for C. elegans development and apparently functions by protecting cells which normally live during development from programmed cell death. As is also described herein, a mutation that constitutively
25 activates ced-9 prevents cells which normally die during development from undergoing programmed cell death, and mutations that inactivate ced-9 result in the deaths of cells which normally survive during development and consequently, in embryo lethality.
30 ced-9 has been shown to function by antagonizing the activities of the cell death genes ced-3 and ced-4. Thus, the C. elegans ced-9 gene appears to act as a binary switch to regulate programmed cell death. Results described herein indicate that many and

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possibly all cells that survive during C. elegans development do so because ced-9 activity prevents them from undergoing programmed cell death.

In addition, a human equivalent of the C. elegans ced-9 gene has been discovered. The deduced amino acid sequence of the ced-9 gene product was found to have about 23% identity and about 47% similarity to the product of the human oncogene bcl-2. This structural similarity, together with previous studies on bcl-2 activity in lymphocytes, strongly suggests that bcl-2 is a human equivalent of ced-9. Applicants further provide methods for identifying other cell death-protective genes from a variety of organisms, including vertebrates (e.g., mammals and particularly humans), invertebrates (e.g., insects), microbes (e.g., yeast), and possibly plants. Furthermore, comparison of ced-9, bcl-2, and other cell death-protective genes and their encoded products provides a way to define key functional features or regions of these genes and gene products. Those features or parts that are conserved between these genes or their gene products are most likely to be functionally important.

Applicants further provide methods and agents for altering the occurrence of cell death in a population of cells and hence, affecting the proliferative capacity and longevity of tissues or organisms. Methods and agents for both decreasing and increasing cell deaths are described. The agents may be all or portions of the cell death-protective genes and encoded products, or derivatives, mimetics, activators or inactivators, or agonists or antagonists of the activity of cell death-protecting genes.

As a result of this work, methods and agents for altering cell death are available for therapeutic or preventive treatment of diseases or conditions

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involving cell death. Methods and agents for reducing cell death are available and are potentially useful for treating disorders and conditions, including those associated with aging, stroke, traumatic brain injury, myocardial infarction, degenerative diseases
5 (including Huntington's disease, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, and Duchenne's muscular dystrophy), and viral and other types of infection (such as with the human immunodeficiency virus or HIV). Methods and agents for increasing cell deaths are also available which are potentially useful for decreasing the growth of or for killing specific cell populations, such as infected cells or autoreactive
10 immune cells. These methods and agents may also be useful for treating diseases or conditions characterized by excessive cell growth or an abnormally low frequency of cell death (e.g., neoplasia and other cancerous growth). Methods and agents which increase cell death are also potentially useful for treating viral, parasitic, and other infections and to kill undesirable organisms, for example, in pest control or biological containment
15 applications.

The invention features an isolated nucleic acid sequence including the nucleotide sequence shown in Figure 2 (SEQ ID NO:1). In addition, the invention features an isolated nucleic acid sequence including the nucleotide sequence encoding a polypeptide of SEQ ID NO:3 which has a glutamic acid to lysine change at amino acid
20 74. The invention also features an isolated nucleic acid sequence, for example, an n3400, n3407, or n3377 nucleic acid sequence, that has a ced-9 loss-of-function mutation and encodes a loss-of-function mutant of the polypeptide of SEQ ID NO:3. Furthermore, the featured nucleic acid sequences may be from a nematode and may be contained in a vector which, in turn, may be in a cell such as a plant or mammalian cell.

25 As used herein, by an "n3400" nucleic acid sequence is meant a nucleic acid sequence shown in Figure 3 that is missing nucleotides 20 to 142 of SEQ ID NO:2 and results in a loss-of-function.

As used herein, by an "n3377" nucleic acid sequence is meant a nucleic acid sequence shown in Figure 3 that has a G to A substitution at position 226 of SEQ ID
30 NO:2 and results in a loss-of-function. For example, this substitution may result in the protein encoded by an "n3377" nucleic acid sequence having a conversion of a glutamic acid to a lysine at position 74 of SEQ ID NO:3.

As used herein, by an "n3407" nucleic acid sequence is meant a nucleic acid sequence shown in Figure 2 that has a G to A substitution at position 2757 of SEQ ID NO:1 and results in a loss-of-function.

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Brief Description of the Drawings

Figure 1 is a diagrammatic representation of a model for ced-9 function. (A) ced-9 is a negative regulator of ced-3 and ced-4 activity. (b) ced-9 acts as a binary switch to regulate programmed cell death. When ced-9 is active, the activities of ced-3 and ced-4 are blocked, and the cell survives. When ced-9 is inactive, ced-3 and ced-4 are activated, leading to programmed cell death.

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Figure 2 shows the nucleotide sequence of the genomic region containing the C. elegans ced-9 gene, with selected restriction sites.

Figure 3 shows the nucleotide sequence of a particular ced-9 cDNA, with selected restriction sites and the predicted translation product.

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Figure 4 shows the predicted amino acid sequence of the Ced-9 protein as deduced from the genomic and cDNA sequences.

Figure 5 shows changes observed in several ced-9 mutants. Shown are changes in the DNA sequence and the resulting predicted change in the protein sequence associated with each mutation.

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Figure 6 shows the optimized alignment of the C. elegans Ced-9 and human Bcl-2 proteins. Identical residues are indicated by vertical bars between the sequences, and similar residues are indicated by one or two dots (. or :), for weak and strong similarity, respectively. A residue that is mutated in the gain-of-function allele n1950 is conserved and has been boxed. Residues mutated in the loss-of-function alleles, n1653ts and n2077, are also indicated by boxes.

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Figure 7 shows the cDNA sequence of bcl-2. The coding sequence is from nucleotides 1459 to 2178, inclusive.

Figure 8 shows the screen for mutations that result in a loss of ced-9 function.

Figure 9 is a diagram of the ced-9 cloning strategy and cosmid rescue. a)

30 Genetic map of the ced-9 region. Relevant genes as well as the approximate position of the N2/RC301 restriction fragment length polymorphisms (RFLPs) used to map ced-9

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are shown. b) Number of recombination events observed between various markers in the unc-69 to unc-49 interval. The nP55 polymorphism did not separate from unc-69 in these experiments, suggesting that unc-69 is to the right, or close and to the left of nP55. c)

- 5 Cosmid rescue of unc-69 and ced-9. Cosmids situated between the nP55 and nP56 RFLPs (recognized by cosmids C15B3 and C38H2, respectively) were injected into unc-69 or unc-69 ced-9/++ animals, and established transgenic lines were tested for rescue of the unc-69 and ced-9 phenotypes. ND: not determined.

- 10 Figure 10 shows the ced-9 locus. a) Restriction map. b) Rescue ability of deletions and point mutations in the locus. c) ced-9 transcripts and location of introns and exons.

- Figure 11 is the ced-9 nucleic acid (SEQ ID NO:2) and Ced-9 amino acid (SEQ ID NO:3) sequence shows the position of various ced-9 mutants including n3400, n3377, and n3407. In addition, this Figure shows several conserved domains, BH1, BH2, BH3, and BH4, that function in protein-protein interactions among Ced-9 polypeptides.
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Detailed Description of the Invention

- Programmed cell death is cell death which occurs during normal development and/or which involves the activities of cell death genes, soome of which may be suicide genes. Programmed cell death is a fundamental aspect of normal development in invertebrates and vertebrates and of vertebrate tissue homeostasis, and may also be an underlying pathological methanism in disorders which involve cell death, including degenerative diseases, stroke, traumatic brain injury, and myocardial infarction, conditions associated with aging, and viral and other types of infection. In addition, some cancers are believed to be caused in part by defects in cell death processes.
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- 25 This invention relates to genes, referred to as cell death-protective genes, which protect cells against programmed cell death by antagonizing the activities of cell death genes (e.g., genes whose activity cause cell death). As described below, a cell death-protective gene from the nematode Caenorhabditis elegans, called ced-9, has been identified, sequenced, and characterized. Mutations which constitutively activate and inactivate ced-9gene function have been
- 30 identified and are also described below. As further

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described below, the deduced amino acid sequence of the ced-9 gene product was found to have about 23% identity and about 47% similarity to the product of the human oncogene bcl-2. The structural and functional similarity of bcl-2 to ced-9 strongly suggests that bcl-2 may be a human equivalent of ced-9 and thus, a cell death-protective gene. Using ced-9 and bcl-2, other cell death-protective genes from a variety of organisms can be obtained. In addition, comparison of equivalent genes and their encoded products, as well as mutational analysis, is expected to indicate key functional features or regions of the genes or gene products. The cell death-protective genes and their gene products are further useful for developing and identifying agents which affect the activity of cell death-protective genes. These agents may be useful for altering (increasing or decreasing) the occurrence of cell death in a cell population or organism, and thus, altering the longevity of the cell population or organism. Further described below are bioassays which are useful for testing and screening for novel cell death-protective genes, mutations in these genes and agents which affect the activity of the genes. Other uses of the invention are also described.

25 The activity of a cell death-protective gene refers herein to the activity of the encoded product(s) of the gene as well as to the gene per se. Thus, agents and mutations which affect the activity of a cell death-protective gene include those which affect the activity of the gene or a product of the gene. The agents may interact with the gene or RNA or protein encoded by the gene, or may exert its effect more indirectly. The agents may affect the level of expression as well as the function of the gene or gene product.

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Genetic Analyses of the *ced-9* Gene

A cell-death protective gene, called *ced-9*, has been identified in the nematode *C. elegans* that functions to prevent cells which normally live during development from undergoing programmed cell death. The *ced-9* gene was defined by a dominant gain-of-function (gf) mutation, called *n1950*, which was mapped to chromosome III. The *n1950* mutation constitutively activates the *ced-9* gene and causes cells which normally die during development to live. Activated *ced-9* prevents programmed cell deaths throughout the animal, and, as shown for certain nerve cells, not only prevents cells from dying, but also generates surviving cells that are sufficiently healthy to function. *ced-9(n1950)* also shows a maternal effect, suggesting that the maternal *ced-9* gene product is contributed to the developing oocyte. Genetic analysis of *ced-9(n1950)* is further described in Example 1 and Table 1 (tables are at the end of the Detailed Description).

Loss-of-function (lf) mutations which inactivate the protective function of *ced-9* and cause cells which normally live during *C. elegans* development to die were also identified. These mutations result in embryonic lethality in the progeny of homozygous animals, indicating that *ced-9* function is essential for development. Four *ced-9(lf)* mutations were isolated, *nDf40*, *n2077*, *n2161*, and *n1653ts*. The lf mutations also show maternal effects. The amount of wild-type *ced-9* product contributed by heterozygous mothers to homozygous *ced-9(lf)* embryos seems to be sufficient to allow these embryos to survive and develop almost normally. As a consequence of this maternal rescue, the lethality that results from an absence of *ced-9* function during early development is apparent only in the second generation. Most of the ectopic cell deaths

observed in the first generation of homozygous ced-9 (lf) animals occur late, during post-embryonic development. It is possible that these late lineages are more seriously affected because dilution or degradation has reduced the amount of maternal ced-9 product to a
5 level at which it cannot effectively protect against cell death. The isolation and genetic analysis of these loss-of-function mutations are further described in Examples 2 and 3 and Table 2.

In addition to the four alleles described above, we isolated another three ced-9 alleles. Sequencing the n3377, n3400, and n3407 loss-of-function alleles showed that all
10 three alleles have molecular changes in the ced-9 nucleic acid sequence (Figure 11). The n3377 allele contains a G to A substitution at position 226 in the ced-9 open reading frame shown in Figure 3 (SEQ ID NO:2). This substitution results in the conversion of a glutamic acid residue to a lysine residue at position 74, of the Ced-9 amino acid sequence shown in Figure 3 (SEQ ID NO:3).

15 Based on a PCR Analysis, the n3400 allele appeared to have a deletion in the ced-9 nucleic acid sequence. We confirmed this result by direct sequencing and showed that the n3400 allele eliminates 121 nucleotides of the ced-9 open reading frame, starting with the fifth codon, and results in a frame shift. The gene product encoded by the n3400 allele is only 11 amino acids long.

20 The n3407 allele contains a G to A substitution as position 2757 of the genomic ced-9 sequence shown in Figure 2 (SEQ ID NO:1). This substitution eliminates the splice acceptor site found at the beginning of exon 3.

As described in Example 4, the ced-9 gene appears to prevent cell death by antagonizing the activities of the cell death genes, ced-3 and ced-4, which have been
25 shown to be required for almost all programmed cell deaths which occur in the development of C. elegans (Ellis and Horvitz, Cell 44:817-829 (1986)).

These results indicate that ced-9 acts as a binary switch to regulate programmed cell death (Figure 1). Remarkably, it seems that many and possibly all cells that survive during C. elegans development do so because ced-9 gene activity prevents

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them from undergoing programmed cell death. Furthermore, cells protected by a constitutively activated ced-9 gene appear to be healthy and to function normally. Thus, ced-9 seems to be a key or master regulatory gene of cell death processes.

5 Sequence Analysis of the ced-9 Gene and Product

The genomic region containing the ced-9 gene was cloned and sequenced, as described in Example 5. Figure 2 shows the nucleotide sequence of this region, including the location of selected restriction sites.

10 Several ced-9 cDNAs representing the same or different transcripts were obtained and sequenced, as described in Example 5. The nucleotide sequence of one of these cDNAs is shown in Figure 3 with restriction sites and the amino acid sequence of the predicted translation product. As shown in Figure 4, ced-9 encodes a 280 amino acid (aa) polypeptide.

15 The gain-of-function mutation, n1950, was also sequenced. As shown in Figure 5, the n1950 mutation, which is responsible for the gain-of-function change in ced-9 activity, is associated with a glycine to glutamic acid change at codon 169. It is likely that this amino acid alteration is the consequence of the n1950 mutation and thus is functionally responsible for the increased activity of ced-9. However, although no other alterations in ced-9 are known to be present in n1950 mutant strains, it remains possible
20 that another alteration exists and that it is this other alteration that is responsible for the gain-of-function change in ced-9 activity. If so, this other amino acid alteration is nonetheless defined by the n1950 mutation and its molecular identity can be determined by DNA sequencing, using established methods. The functional importance of DNA sequence alterations associated with ced-9 mutations can be verified in transgenic C.
25 elegans animals which carry the sequence alteration alone. DNA containing alterations in the wild-type gene can be made by standard methods of in vitro mutagenesis and used to construct the transgenic animals.

The loss-of-function mutation, n1653ts and n2077, were also sequenced and found to be associated with a tyrosine to asparagine change at codon 149 and a glutamine
30 to premature termination at codon 160, respectively.

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Similarity Between ced-9 and a Human Oncogene

Sequence similarity to the ced-9 gene product was discovered in the product of the human oncogene bcl-2 (Tsujiimoto et al., Proc. Natl. Acad. Sci. USA 83:5214-5218 (1986)). Alignment of the two sequences shows 23% identity and 47% similarity between the two proteins (Figure 6). Alignment of the two sequences was generated with the Gap program in the Sequence Analysis Software Package (Genetics Computer Group, Wisconsin), which uses the algorithm of Needleman and Wunsch (J. Mol. Biol. 48:443-453 (1970)) to find the alignment of two complete sequences that maximize the number of matches and minimizes the number of gaps.

bcl-2 is one of a number of genes, of both viral and cellular origin, which are thought to be inhibitors of cell death processes (Vaux et al., Nature 335:440-442 (1988); Henderson et al., Cell 65:1107-1115 (1991); Ciem et al., Science 254:1388-1390 (1991)). Overexpression of bcl-2 prevents or delays the onset of apoptic cell death in both B cells and T cells (Vaux et al. (1988) supra; Nunez et al., J. Immun. 144:3602-3610 (1990); Sentman et al., Cell 67:879-888 (1991); Strasser et al., Cell 67:889-899 (1991)). These cell deaths seem to involve the activities of cell death genes, as gene expression is required for the process (Duke and Cohen, Lymphokine Res. 5:289-299 (1986)). In many tissues in which homeostasis is regulated by cell death, bcl-2 expression occurs in progenitor and long-lived cells (Hockenbery et al., Proc. Natl. Acad. Sci. USA 88:6961-6965 (1991)). The structural similarity of the Bcl-2 protein to Ced-9, together with these previous findings on bcl-2 activity in lymphocytes, suggest that, like ced-9, the bcl-2 gene antagonizes the activities of cell death genes and is required in

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cells that survive to protect them from programmed cell death.

Cell Death-Protective Genes in Other Organisms

As a result of the work described herein, a gene
5 which plays a key role in determining cell death has
been identified, sequenced and characterized. This
invention makes it possible to identify and isolate
equivalent genes in other organisms, including
vertebrates (e.g., mammals and particularly humans),
10 invertebrates (e.g., insects), microbes (e.g., yeast),
and possibly plants. The reasonableness of this
approach has been demonstrated by the structural and
functional similarity of the human gene bcl-2 to ced-9.
As discussed below, there is evidence to suggest that
15 programmed cell death is important in the development
of a variety of organisms and tissues, and that differ-
ent types of cell deaths, whether physiological or
pathological, may share a common mechanism.

Evidence suggests that cell deaths that are mecha-
20 nistically similar to the programmed cell deaths which
occur in the development of C. elegans and other
invertebrates may be very common in vertebrate
development, as well. First, cell deaths that are
similar to the programmed cell death seen in
25 invertebrates were also observed in vertebrates
(Glucksman (1951) supra; Saunders and Fallon, in: Major
Problems in Developmental Biology (25th Symposium of
the Society for Developmental Biology), Lockes (ed.)
Academic Press, New York, 1966, pp. 289-314; Carr and
30 Simpson, Dev. Brain Res. 2:57-162 (1982)). Some of
these cells die shortly after they are born without
obvious differentiation and others have been shown to
be determined to die days before death occurs.

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Second, some vertebrate neuronal cell deaths involve cell interactions and have been thought by some to be different from the apparently cell autonomous programmed cell deaths observed in invertebrates.

5 However, even among the cell dependent deaths of these vertebrate neurons, the expression of new genetic information was found to be necessary for cell death, suggesting that a cell death program must be activated. Thus, it is possible that genes similar to those
10 responsible for programmed cell death in C. elegans (ced-3 and ced-4) act in these vertebrate cell deaths as well.

Third, although many cell deaths in invertebrates are invariant and many in vertebrates are variable, the
15 same mechanisms may nonetheless be responsible. Specifically, invariability does not reveal the mechanism of cell death. For example, invariability could be the result of an invariant cell-cell interaction. Thus, the linker cell in C. elegans males
20 always dies at a certain specific time, but its death requires cell-cell interaction. The invariable programmed cell deaths in moths can, in fact, be prevented by manipulating their hormonal environment or changing cell-cell interactions. These observations
25 argue that the distinction between programmed cell death during invertebrate development and cell death during vertebrate development may be more superficial than real. All of these cell deaths are influenced by genetic factors. Such argument leads to the notion
30 that many and possibly all naturally occurring cell death during development may involve similar mechanisms.

The induction of specific genes has been found during the cell death processes in vertebrates. The
35 induction of TRPM-2 was observed during a variety of

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cell death processes in rodents (Buttayan *et al.*, Mol. Cell. Biol. 9:3473-3481 (1989)). TRPM-2 RNA is at high levels in cells that die during prostate regression in the adult male rat after castration, during renal atrophy following ureteral obstruction in rat, during necrosis of interdigital tissues of the mouse limb bud, and during the chemotherapeutic regression of tumors in rat. Thymocyte cell deaths induced in response to a variety of stimuli can have very similar morphological and biochemical properties, implying the involvement of a single mechanism of cell death. These observations suggest strongly that many types of cell deaths may share a similar mechanism.

In addition, it is possible that of the many human disorders characterized by extensive cell deaths, such as degenerative diseases, stroke, myocardial infarction and traumatic brain injury (for example, see Choi, Neuron 1:623-634 (1988)), some are caused by processes that inactivate or bypass the functions of genes like bcl-2 and ced-9. Furthermore, intracellular calcium has been implicated as a common mediator in a variety of pathological cell deaths, including deaths caused by external injury, toxins, degenerative diseases, ischemia, and hypoxia (Schanne *et al.*, Science 206:700-702 (1979); Farber, Life Science 29:1289-1295 (1981)). Interestingly, amino acid sequence analysis of the C. elegans ced-4 gene product indicates that the Ced-4 protein may contain calcium-binding domains (Yuan, Ph.D. thesis, Harvard University, 1989, p. 139).

30 Uses of the Invention

This invention provides agents and methods based on ced-9, bcl-2, and other cell death-protective genes that are useful for diagnosis and treatment (both therapeutic and preventive) of a variety of disorders

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and conditions involving cell death. The invention is applicable to a variety of organisms, including humans. The genes and their encoded products can be used directly in therapeutics or provide a basis for
5 designing and identifying agents which affect the occurrence of cell death. In addition, mutant forms of these regulatory genes, their encoded products and derivatives of the encoded proteins are available that are potentially useful for treatment.

10 Other cell death-protective genes can be obtained using the methods provided by this invention. As discussed above, it is likely that genes that are structurally and functionally similar to the C. elegans ced-9 gene function in a variety of organisms,
15 including vertebrates (e.g., mammals and particularly humans), invertebrates (e.g., insects), microbes (e.g., yeast), and possibly plants. These equivalent genes have nucleotide sequences similar to portions of the ced-9 gene, or their encoded products have amino acid
20 sequences similar to portions of the ced-9 protein. Equivalent genes also have similar activity to ced-9, in that they protect the cells in which they function from cell death. For example, the human gene bcl-2 was found to be equivalent to ced-9, as described herein.

25 Novel cell death-protective genes can be identified by any number of detection methods which utilize a defined nucleotide or amino acid sequence or antibodies as a probe. The genomic and cDNA nucleotide sequences of ced-9 and the deduced amino acid sequence
30 of the Ced-9 protein are shown in Figures 2, 3, and 4, respectively. The bcl-2 gene and gene products can also be used as probes for cell death-protective genes. The cDNA nucleotide sequence of bcl-2 and the deduced amino acid sequence of the Bcl-2 protein are shown in
35 Figures 6 and 7. For example, nucleic acid (DNA or

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RNA) containing all or part of the ced-9 or bcl-2 genes can be used as hybridization probes or as polymerase chain reaction (PCR) primers. Degenerate oligonucleotides derived from the amino acid sequences of the ced-9 or bcl-2 gene products can be used in these methods. In addition, antibodies, both polyclonal and monoclonal, which bind specifically to the Ced-9 and/or Bcl-2 protein can be produced and used as immunoprobes to screen expression libraries of genes. Databases containing known molecular (nucleotide or amino acid) sequences can also be searched for molecules which are structurally similar to ced-9, bcl-2, or their encoded products.

One strategy for detecting novel cell death-protective genes in various organisms is to initially probe animals which are taxonomically closely related to the source of the probes, for example, probing other worms with a probe derived from ced-9 or probing other mammals with a probe derived from bcl-2. Closely related species are more likely to possess cell death-protective genes or products which are detected with the probe than are more distantly related organisms. These new genes then provide additional sequences with which to probe the molecules of other animals, some of which may share conserved regions with the new genes or gene products but not with ced-9 or bcl-2. This strategy of using related genes in taxonomically closer organisms as stepping stones to genes in more distantly related organisms can be referred to as walking along the taxonomic ladder. However, cell death-protective genes or gene products from a variety of organisms may possess considerable sequence similarity and hence, be identifiable by more direct approaches.

The ced-9 and bcl-2 gene products were found to have 23% identity and 47% similarity. The molecular

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similarity between the ced-9 and bcl-2 gene products is useful, because the similarities between the two proteins reveal which parts or features of these molecules are important for function. For example, an
5 activated bcl-2 may be produced by mutation of the codon which is equivalent to the site of the n1950 mutation in ced-9. More insights on the structure-function relationship of cell death-protective genes are expected to be obtained as more genes equivalent to
10 ced-9 and/or bcl-2 are compared. This knowledge can be used to develop novel molecules which mimic or alter the activity of ced-9, bcl-2 or other cell death-protective genes.

Cell death-protective genes identified as
15 described above can be sequenced by standard methods. Mutated forms of the genes may be identified by such methods, and some of these mutations are expected to constitutively activate and some to inactivate the genes like the n1950 gain-of-function and the loss-of-
20 function mutations in ced-9. Mutationally activated and inactivated forms of cell death-protective genes may be useful for treatment of various disorders, as described further below. In addition, mutagenesis and other sorts of alterations can be performed on the
25 genes and their encoded products to obtain other activated or inactivated proteins.

Mutations may also produce cell death-protective proteins with novel properties. For example, it is conceivable that a cell death-protective gene could be
30 altered such that the gene product actively kills cells, rather than protecting them from cell death, perhaps by activating cell death genes or interfering with the function of wild-type cell death-protective gene products. Mutations and other alterations can be

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accomplished using known methods, such as in vivo and in vitro mutagenesis.

Furthermore, ced-9, bcl-2, or other cell death-protective genes, the corresponding mutant genes, and
5 encoded products can be used to develop agents that activate or inactivate or modulate the activity of the cell death-protective genes. The source of the agents can be such traditional sources as extracts (e.g., bacterial, fungal or plant) and compound libraries, or
10 can be provided by newer methods of rationale drug design. Information on functionally important regions of the genes or gene products, gained by sequence and/or mutational analysis, as described above, would be useful in drug design. The activity of the agents
15 can be verified both by in vivo tests on wild-type, mutant, or transgenic animals containing various forms of ced-9, bcl-2, or other cell death-protective genes, as described below, and by in vitro tests using either cells expressing such genes or the products of these
20 genes directly in biochemical experiments. Potential agents may include all or portions of the ced-9 or bcl-2 genes or gene products (RNA, protein), all or portions of other cell death-protective genes and their encoded products, nucleic acid or peptide derivatives
25 of cell death-protective genes and gene products (e.g., smaller polypeptides and peptides), as well as peptidomimetics, and other molecules which mimic or affect the activity of cell death-protective genes. The agents can also be portions or derivatives of genes which do
30 not by themselves protect cells from programmed cell death but which interact with cell death-protective genes.

This invention further provides bioassays which measure the activity of cell death-protective genes,
35 and hence, are useful for identifying cell death-

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protective genes, for testing mutations in these genes, and for developing agents which mimic or alter cell death-protective activity. The bioassays can be further used to screen expression gene libraries for novel cell death-protective genes from nematodes and other organisms.

In one bioassay, genes or agents are introduced into nematodes to test their effect on cell deaths in vivo. Wild-type, mutant, or transgenic nematodes can be used as appropriate for the expected effect being tested. In one embodiment of the bioassay, transgenic nematodes are produced using sample DNA containing a candidate cell death-protective gene, a mutant cell death-protective gene or a gene library, to observe the effect of the sample DNA on the pattern of cell deaths during development of the nematode, using the methods of genetic analysis described for the ced-9 mutations. For example, a candidate gene can be introduced into a nematode which has a loss-of-function mutation in ced-9 to produce a transgenic nematode. A decrease in cell deaths compared to nontransgenic nematodes would indicate that the sample gene has cell death-protective activity. Similarly, a mutant cell death-protective gene which is inactivated would fail to complement the ced-9 loss-of-function mutation in the transgenic nematode, whereas a constitutively activated gene would decrease the number of cell deaths resulting from the mutation.

In another embodiment of the nematode bioassay, wild-type, mutant, and transgenic nematodes are used to test the effects of specific peptides and other small molecules in order to identify drugs that mimic, increase or decrease cell deaths. For example, wild-type animals can be used to test agents that inactivate or decrease the activity of ced-9 and cause increased

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cell deaths, or that activate or increase the activity of ced-9 and decrease or prevent cell deaths. Mutant or transgenic animals in which ced-9 is underexpressed or inactivated could be used to identify agents that

5 mimic ced-9 in preventing cell death or which act as agonists of cell death-protective activity. Likewise, mutant or transgenic animals in which ced-9 is overexpressed or constitutively activated can be used to identify agents which act as antagonists of cell

10 death-protective activity. Nematodes expressing wild-type ced-9 could be used to identify agents which activate or inactivate the ced-9 gene. The agents may include genes which are not cell death-protective genes but which interact with, regulate, or otherwise affect

15 the activity of ced-9. The agents can be introduced into nematodes by microinjection, diffusion, or ingestion.

Furthermore, agents which affect the activity of other cell death-protective genes, such as bcl-2, can

20 be tested by transgenic animals with a loss-of-function mutation in ced-9. Agents which are non-cell death-protective genes can be tested on cell death-protective genes other than ced-9 by constructing doubly transgenic animals. These animals can be made by

25 crossing a transgenic line which expresses a cell death-protective gene and an inactivated ced-9 gene with a transgenic line which expresses the agent gene.

An in vitro bioassay is also provided. In this bioassay, cultured mammalian cells are used to test

30 genes and agents. Expression gene libraries can also be screened by this method. For example, genes, including genes which are structurally similar to ced-9 or bcl-2, can be introduced into mammalian cells by standard transfection methods to see if they protect

35 from cell death under conditions which induce cell

death, such as exposure to toxins or infection by yeast or bacteria. Mutations which activate or inactivate or otherwise affect cell death-protective activity can be tested. Furthermore, transfected mammalian cells which

5 express a wild-type or mutant cell death-protective gene can be used to test agents which increase or decrease the activity of cell death-protective genes.

Using the above-described nucleic acid and antibody probes and bioassays, the identification and

10 expression of ced-9, bcl-2 or other cell death-protective genes in cultured cells, tissues, and whole organisms can be studied to gain insights into their role in development and pathology. For example, these methods of detection and bioassay can be used to

15 determine if certain mutations in cell death-protective genes, such as bcl-2, are associated with a pathological condition, such as a degenerative disorder.

This invention further provides means of altering

20 or controlling the activity of a cell death-protective gene in a cell or organism, and, thus, to affect the occurrence of cell death. Activity of the regulatory gene can be altered to either increase or decrease cells deaths in a population of cells and, thus, affect

25 the proliferative capacity and/or longevity of a cell population, organ, or entire organism.

ced-9, bcl-2, or other cell death-protective genes, and related and derivative products can be used to protect against cell death of any sort, including

30 degenerative disease, stroke, traumatic brain injury, myocardial infarction, and viral and other types of infection, as well as cell death associated with normal aging. The gene, its encoded RNA, the protein encoded by the gene, or a peptide derived from or related to

35 the gene can be delivered to the affected cells by

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various methods appropriate for the cells or organs being treated, including gene therapy. A non-peptide molecule which mimics, activates, or enhances the activity of a protein encoded by ced-9 or other cell death-protective gene, or polypeptide or peptide derivative, and which is designed on the basis of knowledge of the encoded protein, can also be used. That is, the gene or its product may be used either directly to protect against cell death or as the basis for developing another agent that can function like or increase the activity of the gene or its encoded product.

Mutationally activated forms of the genes can also be used to protect against cell death. Again, the mutated gene, its encoded RNA, the mutant protein encoded by the gene, a peptide derived from or related to the mutant protein, or a non-peptide mimetic, activator or agonist can be used. The n1950 mutation in ced-9 defines one way to make such a gene activated. A mutation equivalent to n1950 can be placed in a cell death-protective gene similar to ced-9 to activate it. For example, a constitutively activated bcl-2 protein might be produced by making a glycine to glutamic acid change at codon 145, as shown in Figure 6, or other sequence alteration equivalent to the one which is responsible for the phenotype of the n1950 mutation in ced-9. (It has not yet been definitively shown that the glycine to glutamic acid alteration of codon 169 of ced-9 is responsible for the activated phenotype of the n1950 mutation. If it is not, the other mutational change(s) in ced-9 responsible for the activation of this gene can be identified as described above and produced in bcl-2 by in vitro mutagenesis to activate bcl-2). The mutant Bcl-2 protein may then be used as a clinically useful molecule or as a basis for developing

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or identifying a clinically useful molecule which protects from cell death.

Alternatively, ced-9, bcl-2, or other cell death-protective genes and their encoded products can be inactivated, or their activity reduced, in order to increase the frequency of cell death. This would be useful, for treating diseases and conditions characterized by an abnormally low frequency of cell death or excessive cell growth, such as neoplastic growth and other cancers. Interestingly, the human cell death-protective gene bcl-2 is also an oncogene, suggesting that cell death processes can be affected in neoplasia. Methods and agents which increase cell death would also be useful for decreasing the growth of or eliminating specific cell populations. For example, populations of autoreactive immune cells may be eliminated or reduced for treating autoimmune disorders. The activity of bcl-2 or other equivalent cell death-protective gene may be inactivated by using single stranded nucleic acid having an antisense sequence which is complementary to the normal transcript of the cell death-protective gene, such as antisense RNA, or DNAs which encode the antisense nucleic acid, or inactivators or antagonists of cell death-protective activity. These agents can be delivered by a variety of methods, including gene therapy. Inactivation of cell death-protective genes may also be useful in treating viral, parasitic and other types of infection, such as human immunodeficiency virus (HIV) infection. A recombinant gene encoding an inactivator or antagonist of cell death-protective activity, such as antisense RNA which is complementary to the transcript of a cell death-protective gene, may be linked to a viral promoter which is specifically activated by a viral protein.

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The recombinant gene is introduced into infected cells. Infected cells containing the viral protein would then be killed and uninfected cells would be unaffected.

Inactivation of cell death-protective genes may also be used to kill organisms for the purpose of biological containment, pest control, or other applications in which populations of undesirable organisms are to be reduced. For example, suicide genes used for biological containment of recombinant bacteria have been reported (Genetic Engineering News, Nov. 1991, p. 13). The suicide genes were engineered to be expressed simultaneously with the desired recombinant gene product so that the recombinant bacteria die upon completion of their task. The present invention provides for construction of recombinant suicide genes encoding antisense RNAs or other inactivators or antagonists of ced-9 or other cell death-protective genes which are useful in organisms in addition to bacteria, for example, in insects, fungi, and transgenic rodents.

Agents which inactivate or inhibit cell death-protective genes can further be used for pest control. For example, many nematodes are human, animal, or plant parasites. Populations of such parasites could be reduced or eliminated by causing their cells to undergo programmed cell death. Parasites present in host animals, including humans, may also be reduced by treatment with agents, such as antisense RNAs, which decrease the activity of a cell death-protective gene specific to the parasite and which leave the host animal unharmed.

The following examples illustrate the invention and are not intended to be limiting in any way.

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EXAMPLE 1

Gain-of-Function Mutation in ced-9

While screening for new C. elegans mutations that affect programmed cell death (Ellis and Horvitz, Development 112:591-603 (1991)), a dominant mutation, n1950, was isolated and genetically characterized, that prevents programmed cell deaths. n1950 was mapped to the right arm of the third chromosome, close to and about 0.05 map units to the right of the mutation unc-69(e587). The n1950 mutation defines a new gene, ced-9 III.

To quantify the effects of the ced-9(n1950) mutation on programmed cell deaths, cells in the anterior half of the pharynx of ced-9(n1950) animals were counted. In wild-type animals there are 49 cell nuclei in this region (Sulston *et al.*, Devl. Biol. 100:64-119, (1983); Albertson and Thomson, Phil. Trans. R. Soc. B275:299-325 (1976)), and in ced-3 and ced-4 animals there are 12-14 additional nuclei (Table 1a). Similarly, in ced-9(n1950) animals there are about 13 extra nuclei in the anterior pharynx. These extra nuclei correspond exactly in position as well as in number to those that fail to die in ced-3 and ced-4 mutants.

Many extra cells survive not only in ced-9(n1950) homozygotes but also in ced-9(n1950)/+ heterozygotes, indicating that the n1950 phenotype is dominant (Table 1b). In addition, the ced-9(n1950) mutation has a maternal effect: about twice as many cells fail to die in heterozygotes generated by mothers carrying at least one copy of the ced-9(n1950) mutation than in heterozygotes generated by homozygous wild-type mothers (Table 1b), suggesting that maternal ced-9 gene product is contributed to the developing oocyte.

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Two observations indicate that ced-9(n1950) is a gain-of-function (gf) mutation. First, n1950 is a rare mutation with dominant effects (only one allele was recovered in a screen of over 24,000 haploid genomes (Ellis and Horvitz (1991) supra), which is a frequency about 10-fold lower than that at which typical loss-of-function mutations are recovered (Brenner, Genetics 77:71-94 (1974); Meneely and Herman, Genetics 92:99-115 (1979); Greenwald and Horvitz, Genetics 96:147-164 (1980)). Second, a deletion (nDf40) that removes the ced-9 gene does not have a dominant effect on cell death (Table 1b).

To study the effects of ced-9(n1950) on programmed cell deaths in regions other than the anterior pharynx, it was determined whether n1950 could prevent the accumulation of cell corpses in ced-1 and ced-5 mutants. In wild-type animals, dying cells are rapidly engulfed and degraded by a neighboring cell. In ced-1 and ced-5 mutants, this engulfment process is blocked, leading to an accumulation of undegraded cell corpses that can easily be seen in young larvae (Hedgecock et al., Science 220:1277-1279 (1983); Ellis et al. Genetics 129:79-94 (1991)). Mutations that inactivate ced-3 or ced-4 block programmed cell death and therefore prevent the accumulation of dead cells in ced-1 or ced-5 animals (Ellis and Horvitz, Cell 44:817-829 (1986)). Similarly, very few corpses appear anywhere in ced-1; ced-9(n1950) or ced-9(n1950); ced-5 double mutants (Table 1c). Thus, the ced-9(n1950) mutation, like mutations in ced-3 and ced-4, prevents programmed cell deaths throughout the animal.

The effects of ced-9(n1950) on the survival and function of a specific pair of nerve cells, the HSNs (hermaphrodite-specific neurons) were also studied (Trent et al., Genetics 104:619-647 (1983); White et

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al., Phil. Trans. R. Soc. B311:1-340 (1986); Desai et al., Nature 336:638-646 (1988); Desai and Horvitz, Genetics 121:703-721 (1989)). The two HSN neurons innervate the vulval muscles and control egg-laying by hermaphrodites. Mutations in the gene eql-1 cause these cells to undergo programmed cell death, resulting in egg-laying defective animals (Ellis and Horvitz, Cell 44:817-829 (1986); Trent et al., (1983) supra; Desai and Horvitz (1989) supra). Mutations in ced-3 and ced-4, which block programmed cell death, prevent the HSNs from dying in eql-1 mutants and suppress the egg-laying defect (Ellis and Horvitz (1986) supra). Similarly, the HSNs are present in ced-9(n1950); eql-1 double mutants, and egg-laying by these animals is normal (Table 1d). Thus, ced-9(n1950), like the ced-3 and ced-4 mutations, not only prevents cells from dying but, at least in this case, also generates surviving cells that are sufficiently healthy to function.

Methods

The data presented in Table 1 were obtained as follows. Cell survival was quantified by counting the cells in the procorpus and metacarpus, which together constitute the anterior half of the pharynx (Albertson and Thomson, Phil. Trans. R. Soc. B275:299-325 (1976)). In wild-type animals there are 49 cell nuclei in this region. Cells that die are generated in characteristic positions (Sulston et al., Devl. Biol. 100:64-119 (1983)), making it easy to identify and count cells that have failed to die. The genotypes of animals studied for Table 1a were as shown.

The complete genotypes of the animals studied for Table 1b were, from top to bottom: wild-type (N2), non-Unc progeny of eT1 unc-36/nDf40 dpy-18 males crossed with unc-36 hermaphrodites, non-Unc progeny of

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n1950 males crossed with unc-69 hermaphrodites, Unc Dpy progeny from n1950/unc-69 dpy-18 hermaphrodites, non-Lon non-Dpy progeny from dpy-17 lon-1/n1950 dpy-18 hermaphrodites, Unc-49 progeny from unc-69/n1950 unc-49.

- 5 heterozygous hermaphrodites, non-Unc progeny of wild-type (N2) males crossed with unc-69 n1950 hermaphrodites, and n1950 self-progeny from n1950 homozygous hermaphrodites.

For the pharyngeal and head corpses in Table 1c,
 10 only young larvae with four cells in the gonad, that is, between hatching and the middle of the first larval stage, were scored (Kimble and Hirsh, Dev. Biol. 70:396-417 (1979)). For ventral cord corpses (descendants from the blast cells P9-P12) and for tail
 15 corpses, third larval stage animals were scored. Extra cells are the number of extra cells among the descendants of P9, P10, and P11. The divisions of these blast cells generate four programmed cell deaths in the wild-type (Figure 6a).

- 20 In Table 1d, HSN missing (%) is the percent of missing or grossly displaced HSN neurons. Only first or second larval stage animals were scored. There are two HSNs per animal, one on each side (White et al., Phil. Trans. R. Soc. B 311:1-340 (1986)). To score egg
 25 laying, staged worms were grown at 20°C. Animals were observed using a dissecting microscope on the second day of adulthood, and those bloated with late-stage eggs were considered egg-laying-defective (Trent and Horvitz, Genetics 104:619-647 (1983)). The alleles
 30 used were: ced-1(e1735), ced-3(n717), ced-4(n1162), ced-5(n1812), ced-9(n1950), dpy-17(e164), dpy-18(e364), eql-1(n478sd, ts), lon-1(e1820), unc-36(e251). eT1(e873), a translocation chromosome with a breakpoint that disrupts unc-36 gene function, prevents crossing
 35 over on the right arm of chromosome III (Rosenbluth and

Baillie, Genetics 99:415-428 (1981)). nDf40 is a new deficiency which was isolated as a cis-acting suppressor of n1950.

5 Animals were anaesthetized with 30 mM NaN₃ (Avery and Horvitz, Cell 51:1071-1078 (1987)) and observed using Nomarski optics microscopy (Sulston and Horvitz, Devl. Biol. 56:110-156 (1987)). Average numbers are shown with, if appropriate, their 95% confidence limits, as determined by the t-test using the StatViewII program (Abacus Concepts, Berkeley, California).

10

EXAMPLE 2

Isolation of ced-9(lf) Mutations

Because the ced-9(n1950) mutation causes a gain of gene function (see above), mutations that reduce or
15 eliminate ced-9 activity (ced-9 loss-of-function (lf) mutations) were isolated by screening for cis-dominant suppressors of ced-9(n1950). Second mutations in ced-9 were expected to be isolated which could suppress the dominant effects of n1950 by inactivating ced-9 (Figure
20 7). After screening 9,000 haploid genomes, three candidate suppressor mutations were isolated which were tightly linked to ced-9(n1950). One of these mutations, nDf40, behaved genetically as a large deletion (see below), indicating that the screening procedure
25 should allow the isolation of mutations that completely inactivate ced-9. The other two mutations, n2077 and n2161, seem likely to be ced-9 loss-of-function alleles: these two mutations failed to complement each other while complementing recessive mutations in all
30 known genes in this region. The n2077 and n2161 mutations mapped within 0.1 map units of the original n1950 mutation, and were obtained at a frequency of about 3×10^{-4} per haploid genome, which is comparable to that

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for loss-of-function mutations in other C. elegans genes (Brenner, Genetics 77:71-94 (1974); Meneely and Herman, Genetics 92:99-115 (1979); Greenwald and Horvitz, Genetics 96:147-164 (1980)).

5 It was then determined that another mutation, n1653ts, which was previously isolated in an unrelated screen for mutants with displaced or missing HSN neurons (Desai et al., Nature 336:638-646 (1988)), was also a ced-9(lf) allele. n1653 was shown to be allelic
10 to n2077 and n2161 based on its position on the genetic map, the similarity of its phenotype at restrictive temperature to the phenotypes of n2077 and n2161 mutants, and its failure to complement n2077 and n2161. Programmed cell deaths occurred normally in n1950
15 n2077/++ and n1950 n2161/++ animals, but were blocked in n1653/n1950 trans-heterozygotes. The results of this cis-trans test demonstrate that the allelic mutations n2077, n2161 and n1653 are in the ced-9 gene, which is defined by the mutation n1950, rather than in
20 a closely linked gene.

Methods

The screen for mutations that resulted in a loss of ced-9 function (see Figure 7) was performed as follows. The semidominant mutation eql-1(n487sd)
25 causes the two HSN neurons to die by programmed cell death, so that the animal bloats with eggs (Ellis and Horvitz, Cell 44:817-829 (1986); Trent et al., Genetics 104:619-647 (1983). Because ced-9(n1950) dominantly suppresses eql-1(n487) by preventing the deaths of the
30 HSN neurons, only animals that do not have ced-9(n1950) function will bloat with eggs as a result of the eql-1 mutation. Such egg-laying defective animals were screened by mating eql-1(n487) V males either with unc-69(e587) ced-9(n1950) III; unc-10(e102) xol-1(y9) dpy-

6(e14) X hermaphrodites or with unc-69(e587) ced-9(n1950) dpy-18(e364) III; lon2(e678) xol-1(y70) X hermaphrodites. Egg laying-defective cross-progeny were picked and their progeny examined for any unusual phenotype. The xol-1 mutation on the X chromosome causes male lethality (Miller *et al.*, *Cell* 55:167-183 (1988) and so prevents mating among F₁ animals, which would complicate genetic analysis of new mutations. The unc-69, dpy-18, lon-2, unc-10, and dpy-6 mutations were used as closely linked genetic markers to identify the chromosomes carrying the ced-9 and xol-1 mutations. General genetic methods and techniques for mutagenesis with ethyl methanesulphonate are described in Brenner, *Genetics* 77:71-94 (1974). Two-factor mapping experiments showed the new mutations n2077 and n2161 to be tightly linked to ced-9(n1950). This screen also generated nDf40, a deficiency that fails to complement unc-69, ced-9, unc-49 and several adjoining genes. The loss-of-function mutation ced-9(n1950 n2077) complements the nearby mutations unc-50(e306), ooc-4(e2078) and emb-25(q45ts); ced-9(n1653ts) complements unc-69(e587). The ooc-4 mutation causes a defect in oogenesis, resulting in hermaphrodite sterility. The mutation emb-25(q45ts) is described in Cassada *et al.*, *Dev. Biol.* 84:103-205 (1981). All other mutations are described in Brenner (1974) *supra*.

EXAMPLE 3

The ced-9(lf) Alleles Cause Ectopic Cell Deaths

Animals homozygous for ced-9(lf) mutations show several defects (Table 2). Most obviously, homozygous ced-9(lf) mutants derived from ced-9(lf)/+ heterozygous mothers hatch and grow to normal size, but generate very few eggs (partial sterility), all of which

eventually die, usually during embryogenesis (maternal effect lethality). Furthermore, such first generation ced-9(lf) animals lack many cells normally present in wild-type animals, resulting in a number of additional defects. For example, many ventral cord motor neurons involved in the control of movement are missing, resulting in uncoordinated body movement. The HSN neurons are missing in hermaphrodites, causing an egg-laying defect (Table 2). Similarly, cells are absent from the male tail, resulting in missing or deformed rays (Table 2). Furthermore, several neurons are sometimes missing from the lumbar ganglion, although their absence does not result in an obvious behavioral abnormality.

To determine why cells are missing, ced-9(lf) animals were observed as they developed. The pattern of cell divisions in wild-type C. elegans is highly reproducible among individuals, and deviations from the normal cell lineage can be identified (Sulston and Horvitz, Dev. Biol. 56:110-156 (1977); Sulston et al., Dev. Bio. 100:64-119 (1983); Kimble and Hirsh, Dev. Biol. 70:396-417 (1979); Sulston et al., Dev. Biol. 78:542-576 (1980)). The studies revealed that many cells that normally survive in wild-type animals instead undergo programmed cell death in ced-9(lf) animals.

For example, the divisions of the 12 ventral cord blast cells P1-P12 (collectively called Pn) were monitored. During the first larval (L1) stage, each P cell divides to generate an anterior daughter (Pn.a) that is a neuroblast and a posterior daughter (Pn.p) that is a hypodermal blast cell. The Pn.a cells then follow identical patterns of divisions to generate motor neurons involved in locomotion (Sulston and Horvitz (1977) supra; White et al., Phil. Trans. R.

Soc. 311:1-340 (1986); Chalfie and White, in: The Nematode Caenorhabditis elegans (eds. Wood et al.), pp.337-391 Cold Spring Harbor Laboratory Press, New York, 1988). Numerous ectopic cell deaths were
5 observed in all Pn.a lineages of ced-9(lf) animals, and frequently all descendants of the Pn.a neuroblasts died.

Ectopic programmed cell death was also observed in the ray lineages. Rays are simple sensory structures
10 located in the male tail, which is used for copulation. Each of the 18 rays arises from a single ray precursor cell (Sulston and Horvitz (1977) supra). Many ectopic cell deaths occurred in the ray lineages of ced-9(lf) males. These ectopic deaths often eliminated the ray
15 structure cell, which is required for ray formation (Sulston and Horvitz (1977) supra). Thus, these deaths account for the absence of rays in ced-9(lf) males.

To determine the cause of the maternal-effect lethality of ced-9(lf) mutations, the embryonic cell
20 lineages of the progeny of ced-9(lf) animals were studied. Embryos generated by mothers homozygous for the weak allele ced-9(n1950 n2161) usually arrested during the early stages of embryo elongation (about 450 minutes after fertilization; Sulston et al. (1983)
25 supra), although there was some variation from animal to animal. These embryos developed normally to about the 200-cell stage, at which point extensive ectopic cell deaths began to appear. These ectopic cell deaths were morphologically similar to the cell deaths that
30 occur during normal C. elegans development and that also first appear at this stage (Sulston et al. (1983) supra). The cell lineage of a single ced-9(n1950 n2161) embryo was analyzed using a 'four-dimensional'-
35 microscope (which allows time-lapse recording of multiple focal planes of a specimen. 49 of the cells

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that died (more than 100 cells died eventually) were identified. Of these 49 dying cells, 45 normally survive in the wild-type. These 45 ectopic deaths prevented the generation of 78 cells, 50 of which would have been neurons or glial cells. Mothers of hypodermal cells and of muscle cells, also died. No obvious pattern to the ectopic cell deaths could be discerned. Many of these deaths involved cells that in the wild-type do not generate any descendants that die. Therefore, these deaths were not simply consequences of premature activation of the pathway for programmed cell death.

Embryos from mothers homozygous for the strong allele ced-9(n1950 n2077) were also studied. The n2077 mutation probably results in a complete or nearly complete inactivation of ced-9, because n2077 behaves like the ced-9 deletion nDf40 when placed in trans to each of the ced-9 alleles (Table 2). Surprisingly, the defects and terminal phenotype associated with this allele were quite different from those of n1950 n2161 embryos. The F_2 n1950 n2077 embryos arrested much earlier in development, with different individuals having from a few dozen to a few hundred cells at most. The embryos invariably looked sick, with swollen cells and abnormal granules in the cytoplasm. Furthermore, cell divisions were slow and asynchronous. In those rare animals that developed sufficiently far before arresting, cell corpses started to appear at about the same stage as in n1950 n2161 embryos. The lineage of a single ced-9(n1950 n2077) embryo was followed with the four-dimensional-microscope. This embryo arrested with 57 cells. Nothing resembling a programmed cell death was observed. However, blocks in mitosis and cytokinesis were apparent, with incomplete cytokineses resulting in the formation of several binucleate cells. It

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is not known whether these defects in cell divisions and the general sickness are effects of a lack of ced-9 function in the embryo or are secondary consequences of abnormalities in the maternal germline. Because all of these defects are completely suppressed by mutations in ced-3 or ced-4 (see below), it seems likely that they are a consequence of the ced-9(n1950 n2077) allele rather than of another mutation carried in this strain. It is likely that these defects are caused by the ectopic activation of the pathway for programmed cell death in the maternal germline. Alternatively, the three genes ced-9, ced-3 and ced-4 might act not only in programmed cell death but also in an aspect of early C. elegans development that is unrelated to programmed cell death.

Methods

The loss of ced-9 function results in ectopic cell deaths. Cell lineages of the ventral cord blast cells P1-P12 in the wild-type (Sulston and Horvitz, Dev. Biol. 56:110-156 (1977)) and cell lineages of P1-P12 in a ced-9(n1950 n2077) hermaphrodite progeny of a qC1/unc-69 ced-9(n1950 n2077) heterozygous mother were studied. The exact pattern of cell deaths varied slightly among the three mutant animals studied. Ray lineages in the wild-type (Sulston and Horvitz (1977) supra; Sulston et al., Dev. Biol. 78:542-576 (1980)) were examined, as well as cell lineages of the left and right R cells, R4L-R9L and R4R-R9R, respectively, in a single ced-9(n1950 n2077) male progeny of a qC1/unc-69 ced-9(n1950 n2077) heterozygous mother crossed with males of identical genotype. The left and right R1-R3 cell lineages were not followed in this particular animal. As in the ventral cord lineages, the exact pattern of ectopic cell deaths varied among the three

mutant animals studied. Also examined were male tails. Unc-69 male tails had nine rays on each side. The male tail of a particular unc-69 ced-9(n1950 n2077) animal has only three rays on the left side and five on the right side. A ced-4 unc-69 ced-9(n1950 n2077) male tail showed 18 rays. In wild-type embryos, 350 minutes after fertilization, only a few cell corpses could be seen in ventral view. In ced-9(n1950 n2161) embryos, generated by a ced-9(n1950 n2161) mother, many corpses could be seen 350 minutes after fertilization.

Cell lineages were followed using Nomarski optics microscopy (Sulston and Horvitz (1977) supra). Four-dimensional-microscopy of embryos was done as follows: freshly fertilized embryos were mounted for observation on 5% agar pads in a drop of M9 or egg salts (Sulston et al., Dev. Biol. 100:64-119 (1983)). Pictures of the developing embryos were taken in 18 focal planes (roughly 1 μ m apart) at 30 second intervals using an apparatus developed by J. G. White and stored on a 12-inch optical video disk for subsequent analysis.

The data for Table 2 were obtained as follows. For Table 2a, the numbers of eggs laid by first generation ced-9(lf) hermaphrodites and the stages of development at which the progeny of these hermaphrodites arrested were examined. First generation hermaphrodites were transferred to fresh plates every 12 hours, and the number of eggs they laid were counted. Note that the absence of HSNs retards but does not prevent egg-laying (Trent et al., Genetics 104:619-647 (1983)), so that the sterility observed in ced-9(lf) animals as reflected by the number of eggs laid per animal cannot be only an effect of the defect in egg release. For example, eql-1(n487sd, ts) animals, which are egg laying-defective because they lack HSNs, nonetheless lay an average of 204 eggs

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(Desai and Horvitz, Genetics 121:703-721 (1989)). Egg-laying defective ced-9(lf) animals do, however, fertilize a few eggs that are never laid. For example, although wild-type hermaphrodites lay all fertilized eggs within 4 days of reaching adulthood, by the seventh day ced-9(n1950 n2077) hermaphrodites still had 1.7 ± 1.3 eggs (number of broods = 12) remaining in utero, and egg-laying defective ced-9(n1950 n2161) hermaphrodites had 30 ± 23 eggs (number of broods = 6). The number of eggs laid by ced-9(lf) animals therefore usually slightly underestimates actual brood size. The percent of eggs laid that hatched within 48 hours of removal of the mother were examined; wild-type eggs hatch about 14 hours after fertilization (Sulston and Horvitz, Devl. Biol. 100:64-119 (1983)). The percent of hatched progeny that failed to develop past the first (L1) larval stage within 6 days of hatching were also examined; wild-type larvae remain in the L1 stage for about 12 hours (Sulston and Horvitz, Devl. Biol. 100:64-119 (1983)).

For Table 2b, the percent of animals defective in egg-laying was scored as in Table 1. Note however that for some genotypes (marked †) a significant fraction of the animals could not be scored accurately for egg-laying capability because of the small number of eggs they generated. Egg-laying defective ced-9(lf) animals do lay eggs in the presence of serotonin (assayed as in Trent et al., Genetics 104:619-647 (1983)), suggesting that the serotonergic HSN neurons are defective or absent.

For Table 2c, young adult males were anaesthetized in 30 mM NaN_3 , placed on their backs and observed using Nomarski optics. All strains were homozygous (nDf40 strains were hemizygous) for the closely linked mutation unc-69(e587), which facilitates identification

of the chromosome carrying the ced-9 mutation. All ced-9(lf) were maintained as heterozygous stocks balanced by the chromosome III balancer qC1. The nDf40 chromosome was marked with dpy-18(e364). nDf40 fails to complement both ced-9 and unc-69. For the n2161/n2077 and n1653/n2077 trans-heterozygotes, the maternally-inherited n2077 chromosome was marked with the dpy-18(e364) mutation to distinguish self- from cross-progeny. The HSN counts for the n2161/n2077 and n1653/n2077 genotypes were not determined because of the difficulty of scoring the Dpy phenotype in early larvae. The ced-9(+)/Df larvae that arrested as L1s did so as a consequence of the unc-69(e587) mutation, which decreases brood size and results in an incompletely penetrant L1-arrest phenotype when heterozygous with nDf40; by contrast, nDf40/unc-69(+) animals do not arrest development as L1 larvae.

EXAMPLE 4

The ced-9 Gene Antagonizes ced-3 and ced-4

If the defects associated with a loss of ced-9 function are caused entirely by the aberrant activation of the programmed cell death pathway, then mutations that prevent the process of programmed cell death might be able to suppress these defects. To test this hypothesis, double mutants were constructed using ced-9(n1950 n2077) and mutations in either ced-3 or ced-4, two genes required for programmed cell death (Ellis and Horvitz, Cell 44:817-829 (1986)). Mutations in ced-3 or ced-4 completely suppressed all defects observed in ced-9(n1950 n2077) animals (Table 3, Figure 8g). Similar results were obtained for ced-9(n1950 n2161) and ced-9(n1653ts). These observations suggest that the defects seen in ced-9(lf) animals are indeed caused

by the activation of the programmed cell death pathway. Furthermore, if these three genes are part of a regulatory pathway, these results indicate that ced-9 acts before ced-3 and ced-4, because the activities of these genes are required for ced-9(lf) mutations to have their effects.

Methods

The data for Table 3 were obtained as follows. The numbers of eggs laid were determined as described for Table 2. Viable progeny are the number of progeny that grew to the fourth larval (L4) stage within 10 days of hatching (this value includes a few animals that developed from eggs that hatched internally); wild-type larvae reach the L4 stage within 2 days. (Sulston and Horvitz, Devl. Biol. 56:110-156 (1977)). Confidence limits (95%) were determined as in Table 1. Note that ced-3 and ced-4 are able to suppress the ced-9(lf) zygotic defects in a semidominant fashion: animals homozygous for ced-9(n1950 n2077) but carrying only one wild-type copy of either the ced-3 or ced-4 genes showed milder zygotic defects than did animals with two wild-type copies of both genes, suggesting that lowering ced-3 or ced-4 activity can compensate for lower levels of the Ced-9 protein in first generation ced-9(lf) animals. However, one copy of ced-3 or ced-4 is not sufficient to suppress the maternal-effect lethality: all the viable progeny generated from ced-9(lf); ced-3/+ mothers were homozygous for the ced-3 mutation. Double mutants were also constructed between ced-9(n1950 n2077) and ced-3(n718), ced-3(n1040), ced-3(n1128), ced-3(n1949), ced-4(n1894), or ced-4(n1920), and between ced-9(n1950 n2161) and ced-3(n717), ced-4(n1162), ced-4(n1894), or ced-4(n1920). All of these double mutants were both

viable and fertile, showing that the suppression of the ced-9(lf) defects by ced-3 and ced-4 is not allele-specific.

EXAMPLE 5

5 Cloning and Sequencing the ced-9 Gene and cDNACloning of ced-9

ced-9 was genetically mapped to the right arm of chromosome III, approximately 0.05 map units to the right of unc-69. This position placed ced-9 between the two cloned genes lin-12 and tra-1. The whole interval between these two genes, corresponding to approximately 2 Mb, had previously been cloned as part of the C. elegans physical mapping effort (Coulson et al., Proc. Natl. Acad. Sci. USA 85:4397-4401 (1986); Coulson et al., Nature 335:184-186 (1988); Coulson et al., BioEssays 13:413-417 (1991)). To narrow down the region containing ced-9, ced-9 was mapped with respect to a series of restriction fragment length polymorphisms (RFLPs) between the common laboratory strain Bristol N2, and RC301, a strain isolated from the wild near Freiburg, Germany.

Mapping of ced-9 with respect to these RFLPs (Figure 9b) localized the gene to a roughly 60 Kb interval located between np55 and np56. Cosmids from this region were then tested for their ability to rescue the ced-9(lf)-associated defects. Three overlapping cosmids were found to be able to rescue ced-9 (Figure 9c). Various fragments from the region common to all three cosmids were subcloned into plasmids and tested for rescue activity. A 4.7 Kb SacI-XhoI fragment was identified in this manner that efficiently rescued both the sterility and the lethality of ced-9(n1950 n2077) mutants. Further

deletions into this fragment from either the right or the left abolished or greatly diminished the rescuing the activity of the fragment (Figure 10b).

ced-9 cDNAs

5 A 4.2 Kb ClaI-XhoI fragment was used to probe a C. elegans cDNA library. Three distinct classes of cDNAs were isolated, corresponding to the 0.75 Kb, 1.3 Kb, and 2.2 Kb transcripts identified on Northern blots. One cDNA of each class was sequenced. The deduced
10 intron/exon structure of the three classes of cDNAs is shown in Figure 10c. Both the 0.75 Kb and 2.3 Kb cDNAs sequenced had SL1 trans-spliced leaders at their 5' ends, suggesting that full-length cDNAs were isolated. The three cDNA classes are related to each other in an
15 unusual way: the 0.75 and 2.2 Kb transcripts share the same 5' end and open reading frame and are predicted to encode identical proteins. The 1.3 Kb and 2.2 Kb transcripts are predicted to share the same polyadenylation site.

20 METHODS:

Mutations and Strains

All mutations were generated in a Bristol N2 background, which was used as the standard wild-type strain, except where noted. The following mutations
25 were used:

LGIII: unc-69(e587), ced-9(n1950dm), ced-9(n1950 n2077), ced-9(n1950 n2161), unc-49(e382).

Mutations other than ced-9 are described in (Brenner, Genetics 77:71-94 (1974)). The ced-9(n1950 n2077) and ced-9(n1950 n2161) mutations were maintained
30 as balanced strains over the LGIII balancer gC1, which carries the mutations dpy-19(e1259ts.mat) glp-1(q339)

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Strains were maintained as described (Brenner, 1974 supra). All strains were grown at 20°C.

RFLP Mapping

5 Various cosmids from the lin-12 to tra-1 interval were tested for their ability to detect RFLPs between the common laboratory strain N2 and various strains isolated from the wild and known to contain a large number of transposon-induced RFLPs. The position of ced-9 was then determined relative to these markers as described (Ruvkun et al., Genetics 121:501-516 (1989)).
10 Briefly, N2/RC301 recombinants in the ced-9 region were obtained by mating RC301 males with unc-69(e587) ced-9(n1950) unc-49(e382) hermaphrodites to generate unc-69 ced-9 unc-49 [N2]/+++[RC301] heterozygotes. From these
15 heterozygotes, Unc-49 non-Ced-9 non-Unc-69 and Unc-49 Ced-9 non-Unc-69 recombinants were cloned, homozygosed for the recombinant chromosome, and the genotype of the various RFLP loci analyzed by genomic Southern blot analyses.

20 Germline Transformation of ced-9 Mutants

DNA to be tested for ced-9 rescue activity were microinjected into the mitotic germline of hermaphrodites according to the method developed by Mello and colleagues (Mello et al., EMBO J. 10:3959-
25 3970 (1991)). The relevant DNA was injected at a concentration of 5-25 µg/ml. pRF4, a plasmid containing a dominant rol-6 mutation, was co-injected as a dominant marker to identify transgenic animals. Since ced-9(lf) mutants are almost sterile and produce
30 only dead progeny, heterozygotes of genotype gC1 dpy-19(e1259)/unc-69(e587) ced-9(n1950 n2077) were injected, where the unc-69 was used as a linked marker

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to identify the ced-9 chromosome. Non-Unc non-Dpy Roller Fls were picked to establish stably transmitting roller lines. From these, Roller Unc-69 animals were picked and tested for rescue of the ced-9(lf)-
5 associated sterility and maternal effect lethality. A clone was considered to rescue if a stable homozygous line of genotype unc-69 ced-9(lf) III; array could be established.

Molecular Biology

10 Standard molecular biology protocols (see (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1989) was followed except where noted. All plasmid subcloning was done into pBluescript vectors (Stratagene).

15 The 4.7 Kb genomic SacI-XhoI fragment with rescuing ability was subcloned into pBluescript II, (Stratagene) and both strands were sequenced using the ExoIII-S1 nested deletion method and T7 polymerase (Sequenase, USB) following the protocol suggested by
20 the manufacturer.

A 4.2 Kb ClaI-XhoI rescuing genomic fragment was used to probe a λ cDNA library. From approximately 300,000 plaques, 11 cDNAs were isolated. The sequences present at the ends of the inserts were determined for
25 all 11 cDNAs. 8 cDNAs corresponded to the 0.75 Kb cytochrome b560-like transcript, two (one incomplete) were from the 1.3 Kb ced-9 transcript, and one corresponded to the 2.2 Kb "fusion" transcript. One cDNA from each class was then completely sequenced (one
30 strand only).

TABLE 1
The Gain-of-Function Allele *ced-9(n1950)* Prevents Programmed Cell Deaths

| a. <i>ced-9(n1950)</i> prevents programmed cell deaths | | | No. of Animals |
|--|----------------------------------|------------------------------------|-------------------|
| Maternal genotype | Zygotic genotype | Extra cells in anterior pharynx | |
| <i>ced-3/ced-3</i> | <i>ced-3/ced-3</i> | 12.5±0.7 | 30 |
| <i>ced-4/ced-4</i> | <i>ced-4/ced-4</i> | 13.9±0.5 | 40 |
| <i>ced-9(n1950)/ced-9(n1950)</i> | <i>ced-9(n1950)/ced-9(n1950)</i> | 13.3±0.6 | 45 |
| b. <i>ced-2(n1950)</i> is a dominant gain-of-function mutation and shows a maternal effect | | | |
| Maternal genotype | Zygotic genotype | Extra cells in anterior pharynx | No. of Animals |
| <i>+/+</i> | <i>+/+</i> | 0.03±0.05 | 60 |
| | <i>Df/+</i> | 0.00 | 50 |
| | <i>ced-9(n1950)/+</i> | 5.3±0.8 | 25 |
| | <i>+/+</i> | 0.2±0.22 | 25 |
| <i>ced-9(n1950)/+</i> | <i>ced-9(n1950)/+</i> | 11.4±0.8 | 30 |
| | <i>ced-9(n1950)/ced-9(n1950)</i> | 13.7±0.5 | 30 |
| | <i>ced-9(n1950)/+</i> | 11.8±0.6 | 30 |
| <i>ced-9(n1950)/ced-9(n1950)</i> | <i>ced-9(n1950)/ced-9(n1950)</i> | 13.3±0.6 | 45 |

c. ced-9(n1950) suppresses the accumulation of cell corpses

| Genotype | Corpses Pharynx | n | Corpses Head | n |
|---------------------|--------------------|------|-----------------|-----|
| Wild-type (N2) | 0 | 50 | 0.0±0.1 | 50 |
| ced-1 | 0.8±0.2 | 100* | 28 | 10† |
| ced-1; ced-3 | 0.02±0.04 | 50 | 0.3±0.1 | 50 |
| ced-1; ced-4 | 0.02±0.04 | 50 | 0.7±0.2 | 50 |
| ced-1; ced-9(n1950) | 0 | 30 | 0.5±0.3 | 30 |
| ced-5 | 3.6±0.6 | 25 | 16±5 | 10 |
| ced-5; ced-3 | 0.1±0.1 | 40 | 0.5±0.2 | 40 |
| ced-4; ced-5 | 0.2±0.2 | 40 | 1.0±0.3 | 40 |
| ced-9(n1950); ced-5 | 0.1±0.1 | 100 | 0.8±0.4 | 25 |

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| Corpses | | | | Extra Cells | |
|-----------|---------|---------|---------|-------------|----|
| P9-P11 | P12 | Tail | P9-P11 | n | n |
| 0 | 0 | 0 | 0 | 0 | 30 |
| 3.5±0.3 | 1.7±0.3 | 1.7±0.3 | 0.4±0.3 | 0.4±0.3 | 30 |
| 0.03±0.07 | 0 | 0.3±0.2 | 3.9±0.1 | 3.9±0.1 | 30 |
| 0.03±0.07 | 0 | 0.3±0.2 | 4.0±0.1 | 4.0±0.1 | 30 |
| 0 | 0 | 0.3±0.2 | 4.0±0.1 | 4.0±0.1 | 30 |
| 3.0±0.5 | 2.2±0.3 | 4.6±0.8 | 0.2±0.2 | 0.2±0.2 | 21 |
| 0.05±0.10 | 0 | 0.4±0.4 | 3.9±0.1 | 3.9±0.1 | 20 |
| 0.05±0.10 | 0 | 1.6±0.5 | 3.9±0.2 | 3.9±0.2 | 20 |
| 0 | 0 | 1.1±0.3 | 3.8±0.1 | 3.8±0.1 | 30 |

d. ced-9(n1950) prevents the deaths of the HSN neurons in egl-1 mutants

| Genotype | HSNs missing (%) | No. of sides | Egg-laying defective (%) | n |
|----------------------------|------------------|--------------|--------------------------|-----|
| Wild-type (N2) | 1 | 250 | 0.4 | 704 |
| <u>egl-1</u> | 99 | 200 | 99 | 447 |
| <u>ced-3; egl-1</u> | 0 | 160 | 0.2 | 599 |
| <u>ced-4; egl-1</u> | 0 | 100 | 0 | 417 |
| <u>ced-9(n1950); egl-1</u> | 0 | 200 | 0 | 417 |

a. The genotypes of animals studied were as shown.

b. The complete genotypes are given in Example 1.

c. Extra cells, number of extra cells among the descendants of P9, P10, and P11.
n, number of animals scored. †Data from Ellis et al., Genetics 122:79-94 (1991).
†Data from Ellis and Horvitz, Cell 44:817-829 (1986).

d. HSN missing (%), percent of missing or grossly displaced HSN neurons. No. of sides, number of sides scored. n, number of animals scored.

Average numbers are shown with, if appropriate, their 95% confidence limits.

TABLE 2

Phenotypes of *ced-9(lf)* Mutants

| Genotype* | <i>ced-9(+)</i> <i>ced-9(+)</i> | | <i>ced-9(+)</i> <i>ced-9(+)</i> | | | | | <i>ced-9(+)</i> <i>ced-9(+)</i> | | <i>ced-9(+)</i> <i>ced-9(+)</i> | | <i>ced-9(+)</i> <i>ced-9(+)</i> | |
|---|---------------------------------|---------|---------------------------------|---------|---------|---------|---------|---------------------------------|------|---------------------------------|------|---------------------------------|------|
| | 20°C | 20°C | 15°C | 20°C | 23°C | 25°C | 20°C | 20°C | 20°C | 20°C | 20°C | 20°C | 20°C |
| (a) Sterility and maternal-effect lethality | | | | | | | | | | | | | |
| Eggs laid per animal | 209±33 | 202±60 | 117±36 | 97±31 | 45±22 | 6.3±4.5 | 23±14 | 40±9 | | | | | |
| Hatching (%) | 99±1 | 75±2 | 12±3 | 2.4±0.7 | 0.4±0.4 | 0 | 0 | 0 | | | | | |
| L1 arrest (%) | 0 | 13±3 | 100 | 100 | 100 | NA | NA | NA | | | | | |
| | n=14 | n=9 | n=23 | n=42 | n=36 | n=60 | n=50 | n=49 | | | | | |
| (b) Egg-laying defect | | | | | | | | | | | | | |
| Egg-laying defective (%) | 0 | 0 | 64±16 | 76±13 | 94±7 | 98±3 | 96±4 | 96±4 | | | | | |
| | n=100 | n=35 | n=23 | n=42 | n=36 | n=60 | n=50 | n=40 | | | | | |
| HSNs missing (%) | 0 | 0 | 77 | 87 | 94 | 95 | 95 | ND | | | | | |
| | n=100 | n=48 | n=118 | n=138 | n=100 | n=130 | n=60 | | | | | | |
| (c) Absence of rays in male tails | | | | | | | | | | | | | |
| Rays per side | 8.9±0.1 | 8.6±0.2 | 8.0±0.3 | 6.6±0.3 | 5.9±0.3 | 5.4±0.4 | 6.0±0.3 | 5.9±0.3 | | | | | |
| | n=68 | n=34 | n=40 | n=40 | n=58 | n=34 | n=62 | n=38 | | | | | |

TABLE 2 (continued)
Phenotypes of ced-9(lf) Mutants

| Genotype* | n1653ts 25°C | n1653ts Df 25°C | n1653ts n1950 n2077 25°C | n1950 n2077 20°C | n1950 n2077 Df 20°C |
|---|-----------------|-----------------------|--------------------------------|---------------------|---------------------------|
| (a) Sterility and maternal-effect lethality | | | | | |
| Eggs laid per animal | 2.7±1.1 | 0 | 0.3±0.4 | 1.6±1.4 | 0.8±1.6 |
| Hatching (%) | 38±24 | NA | 0.7±1.4 | 0 | 0 |
| L1 arrest (%) | 40±36 | NA | 100 | NA | NA |
| | n=26 | n=15 | n=30 | n=20 | n=24 |
| (b) Egg-laying defect | | | | | |
| Egg-laying defective (%) | NA† | NA† | NA† | NA† | NA† |
| HSNs missing (%) | ND | ND | ND | 99 | 100 |
| | | | | n=220 | n=42 |
| (c) Absence of rays in male tails | | | | | |
| Rays per side | 8.6±0.2 | 7.6±0.3 | 8.1±0.3 | 4.6±0.3 | 4.9±0.6 |
| | n=38 | n=44 | n=52 | n=81 | n=26 |

- a. Hatching (%), percent of eggs laid that hatched within 48 hours of removal of the mother.
L1 arrest (%), percent of hatched progeny that failed to develop past the first (L1) larval stage within 6 days of hatching. n, number of broods analysed.
- b. For some genotypes (marked †) a significant fraction of the animals could not be scored accurately for egg-laying capability. n, number of animals scored. HSN missing (%), percent of missing or grossly displaced HSN neurons. n, number of sides scored.
- c. Rays per side, number of rays present per side in the male tail. n, number of sides scored. Confidence limits (95%) were determined as in Table 1. NA, not applicable. *All strains were homozygous (*nDf40* strains were hemizygous) for the closely linked mutation *unc-69* (3587).

TABLE 3
Mutations in *ced-3* and *ced-4* Suppress the Defects Resulting From the Loss of *ced-9* Function

| Genotype* | Sterility and maternal-effect lethality | | Egg-laying defect | | Male tail | |
|--|---|----------------|-------------------|--------------------------|-----------|---------------|
| | Eggs laid per animal | Viable progeny | n | Egg-laying defective (%) | n | Rays per side |
| <i>ced-9</i> (+) | 207±36 | 207±33 | 14 | 0 | 100 | 8.9±0.1 |
| <i>ced-9</i> (n1950 n2077) | 1.6±1.4 | 0 | 20 | NA† | 100 | 4.6±0.3 |
| <i>ced-4 ced-9</i> (n1950 n2077) | 200±19 | 160±20 | 12 | 1 | 84 | 9.0 |
| <i>ced-4</i> | 182±17 | 148±17 | 12 | 0 | 100 | 9.0±0.1 |
| + <i>ced-9</i> (n1950 n2077) | 56±20 | 3.9±2.0 | 7 | 62 | 71 | 7.4±0.3 |
| <i>ced-4 ced-9</i> (n1950 n2077) | 154±29 | 94±22 | 13 | 0 | 100 | 9.0 |
| <i>ced-9</i> (n1950 n2077); <i>ced-3</i> | 178±51 | 146±46 | 6 | 0 | 66 | 8.9±0.1 |
| <i>ced-3</i> | 132±62 | 17±6 | 10 | 18 | 40 | 8.7±0.1 |
| <i>ced-9</i> (n1950 n2077); <i>ced-3</i> / + | | | | | | |

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Viable progeny, number of progeny that grew to the fourth larval (L4) stage within 10 days of hatching (this value includes a few animals that developed from eggs that hatched internally); n, number of broods analysed.

Confidence limits (95%) were determined as in Table 1.

*All strains are homozygous for the closely linked mutation *unc-69*(e587).

†Many animals could not be accurately scored for egg-laying.